J. Microbiol. Biotechnol. (1998), 8(6), 645 - 649

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

Characterization of Glycolipid Biosurfactants from an Isolated Strain of *Pseudomonas aeruginosa* YPJ80

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Received: September 2, 1998

Abstract A glycolipid type of biosurfactants was obtained from a strain which had been isolated from soil. The cell was identified as *Pseudomonas aeruginosa* from taxonomic characteristics and was designated as YPJ80. Thin layer chromatography and deoxyhexose detection tests were done to verify the type of biosurfactant. Critical micelle concentration (CMC) of the surfactant was observed to be 50 ppm and the minimum surface tension was 30.1 mN/m. As an emulsifier, YPJ80 biosurfactant was superior to emulsan in the emulsification of crude Arabian light oil.

Key words: *Pseudomonas aeruginosa*, isolation, glycolipid biosurfactant, emulsification

Biosurfactants are of growing interest in biotechnology industries for several reasons. Firstly, they have unique chemical structures which may have unusual properties beneficial to clean-up of oil spills, to enhanced oil recovery, to drug delivery, and to the cosmetics industry. Secondly, they are produced from microorganisms with a wide diversity of structure which offers the possiblity of isolating highly effective surfactants by screening the structural pool of biosurfactants. Thirdly, biosurfactants are a naturally occuring, biodegradable product with low toxicity. Thus, the release of biosurfactants may be easier to justify to appropriate regulatory agencies than the release of synthetic surfactants [4, 6].

It is well-known that some bacteria, yeasts, and fungi can grow on water-insoluble hydrophobic substrates as the only carbon source by producing extracellular surface active agents or using cell wall-associated surfactants, and the biosurfactants facilitate cells' uptake of hydrocarbons during growth by helping the penetration of insoluble hydrocarbons to the periplasmic space in the form of emulsion [7, 8]. Thus, screening of microorganisms which can grow on hydrophobic substrates as the sole carbon source could be a good start in obtaining useful biosurfactants.

Biosurfactants can be classified into several groups on their chemical structure: glycolipids, lipopeptides, lipopolysacchrides, phopholipid, and fatty acids/neutral lipids. Among them, the glycolipid and lipopeptide surfactants are commonly isolated and the best investigated. In glycolipid surfactants, carbohydrates are combined with long-chain aliphatic acids or hydroxyl-aliphatic acids. Glycolipids usually have molecular weights ranging from 400 to 1,000. A thorough review of the biosurfactant classification and their properties can be found in Lang and Wagner [5].

The purpose of this study was to obtain biosurfactants with high emulsification activity and to evaluate their performance in the emulsification of crude oil. The screening of microorganisms, the identification of the strain, and the characterization of the biosurfactants are described in this paper along with the evaluation of surfactants as emulsifiers.

MATERIALS AND METHODS

Chemicals

Glucose, soybean oil, hexadecane, 2-methylnaphthalene, KH_2PO_4, $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, agar, and NaOH were purchased from Sigma (St. Louis, U.S.A.). Yeast extract and plate count agar were from Difco (Detroit, U.S.A.). HCl and $MgSO_4 \cdot 7H_2O$ were from Oriental Chemical (Japan). All other chemicals used were of reagent grade. Crude Arabian light oil was kindly donated by Dr. Byung-Dae Yoon of KRIBB. Emulsan was a kind gift from Prof. Jeong-Hoe Kim, KAIST.

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Isolation and Cultivation of Strains

More than one hundred of microorganisms were isolated from soils around fuel stations, landfill sites, and streams in Taejon, S. Korea. Soil samples were suspended in 0.9% NaCl solution containing 0.05% SDS and shaken (200 rpm) at 30°C for 24 h. The suspension was properly diluted and plated on a solid agar (17 g/l) medium. The medium contained: $(NH_4)_2SO_4$, 1 g/l; K_2HPO_4, 1.5 g/l; KH_2PO_4, 0.75 g/l; yeast extract, 1 g/l; $MgSO_4 \cdot 7H_2O$, 0.5 g/l; and 20 ml/l of the following solution of trace elements: NaCl (5 g/l), $CaCl_2 \cdot 2H_2O$ (0.5 g/l), $MnSO_4 \cdot 5H_2O$ (0.5 g/l) and $FeSO_4 \cdot 7H_2O$ (0.5 g/l). Trace elements were dissolved in an acidic solution containing 1% (v/v) concentrated HCl. It was then added into the medium after separate sterilization. For a carbon source, 10 g/l of soybean oil was added with 0.4% xanthan to help oil dispersion in the agar medium. Liquid medium was prepared by the same manner as the solid medium except agar was not added, and the pH was adjusted to 7.2 by using 1 N NaOH. Growth was initiated with 2 ml inocula of late-exponential-phase cultures into 20 ml of media in 250 ml flasks. Cultivation was carried out at 32°C with the agitation speed of 250 rpm for 3 days. The carbon source for the preparation of biosurfactants from YPJ80 was glucose (20 g/l) when specific statement on the carbon source was not given.

Selection and Identification

Culture broths were tested for surface tension and emulsification activity, compared with one another, and a strain with the highest activity was selected. Identification of the strain was made based on the taxonomic characteristics listed in Bergey's Manual of Determinative Bacteriology [3]. This strain was identified as *Pseudomonas aeruginosa* and named YPJ80. It was stored at 4°C on plate count agar.

Electron Microscopy Prepearation

Cells were grown on glycerol (20 g/l) for 2 days, centrifuged, and washed with 20 mM citrate-phosphate buffer (pH, 5.3), treated with 2.5% glutaraldehyde for 1 h and then washed extensively with distilled water. The water contained in the specimen was removed by lyophilization. The specimens were glued to aluminium plates, spurted with 15 nm gold, and examined under a scanning electron microscope. For transmission electron microscope, lyophilized cells were embedded in LR White resin (Polysciences, Inc. Warrington, U.S.A.), which was polymerized by 24 h of incubation at 60°C. Ultrathin sections were poststained with 2% uranyl acetate and examined.

Partial Purification

Cells in the culture broth were removed and the pH of the supernatant was lowered to 2.0 by the addition of HCl (6 N). An equal amount of mixed solvent, chloroform: methanol (2:1 v/v), was added to the acidified supernatant for solvent extraction, and the extraction was carried out three times. The solvent was removed by using rotary evaporator. Powdered biosurfactants were dissolved in 20 mM phosphate buffer (pH, 7.0) and used for the thin layer chromatography and the evaluation of emulsification activity in comparison with that of emulsan.

Surface Tension and Emulsification Activity

Surface tension of cell-free culture broth was measured using a Digital Tensiometer K10ST (Kruss, Hamburg, Germany). As a diluent, 20 mM citrate-phosphate buffer (pH, 5.3) was used. The measurement of emulsification activity of biosurfactants (solvent extract) was based on Rosenberg's emulsification test [12]. Hexadecane/2-methylnaphthalene mixture (1:1 v/v) was prepared. The hydrocarbon mixture (0.1 ml) was added to 10 ml of 20 mM citrate-phosphate buffer (pH 5.4) containing an appropriate volume of the biosurfactants solution in a 50 ml flask. After reciprocal shaking (150 strokes per min) for 1 h at 25°C, the resulting emulsion was allowed to stand for 10 min. Its absorbance through 1 cm pathlength was then measured at 620 nm with a Hewlett Packard Spectrophotometer (HP8452, U.S.A.). Emlsification activity was expressed as the absorbance.

Analytical Methods

Detection of deoxyhexose and hexose was made according to Chandrasekaran and Bemiller [2]. A sample (1 ml) was mixed with diluted sulfuric acid (4.5 ml) and boiled for 10 min. After cooling, thioglycolic acid (0.1 ml) was added and the sample was kept in the dark for 3 h. UV absorbances at 400 nm and 430 nm were read and compared with the prepared calibration curve. For thin layer chromatography (TLC), silicagel 60 (F_{254}; Merck Co. Germany) was used. Chloroform:methanol:acetic acid: water (65:25:4, v/v) was used for developing the solvent.

RESULTS AND DISCUSSION

Isolation and Identification

Colonies grown in the presence of soybean oil as the sole carbon source were obtained after the enrichment culture. From more than one hundred type of colonies appearing on solid medium, strains with high emulsification designated as YPJ31, YPJ80, and YPJ146 were selected. Among them, strain YPJ80 showed the highest emulsification activity and yielded the lowest surface tension of the cultivation broth, and was selected for further study (Table1).

The morphological and physiological characteristics of strain YPJ80 are summarized in Table 2. Strain YPJ80 could not grow at 5°C but grew at 42°C. It was found to

Table 1. Bioemulsifier production by selected strains.

Strain	Carbon source	Emulsifying activity, OD_{620}	Surface tension (mN/m)	
			4 times dilution	8 times dilution
YPJ31	Glucose	-	64.1	67.3
	Soybean oil	0.62	34.5	38.5
	Hexadecane	0.52	47.8	49.8
YPJ80	Glucose	0.54	36.1	42.4
	Soybean oil	**1.19**	44.3	51.3
	Hexadecane	-	55.4	59.2
YPJ146	Glucose	0.58	53.3	56.1
	Soybean oil	0.62	37.5	48.2
	Hexadecane	0.21	54.2	57.4

-: not detectable.

Table 2. Morphological and physiological characteristics of YPJ80.

Characteristics	Strains	
	YPJ80	*Pseudomonas aeruginosa*
Gram staining	-	-
Morphology	long rod	long rod
Width of rod	0.4~0.5 μm	0.5~1.0 μm
Length of rod	1.4~1.8 μm	1.5~5.0 μm
Physiology		
Catalase	+	+
Oxidase	+	+
OF test	+/-(O)	+/-(O)
Motility	+	+
Arginine decarboxylase	+	+
Urease	+	+
Nitrate reduction	-	+
Indole	-	-
Methyl Red	+	+
Voges-Proskauer	-	-
Utilization of citrate	+	+
Acid formation from		
glucose	+	+
adonitol	-	-
arabinose	-	-
lactose	-	-
cellobiose	-	-
maltose	-	-
sucrose	-	-
mannitol	-	+
myo-inositol	-	-
salicin	-	-

be gram negative, motile, and capable of fermentation. It showed negative to Voges-Proskauer, indole, nitrate, and starch, while positive to methyl red, citrate, McConkey, gelatin, casein, KCN, and aesculin. These characteristics are in good accordance with those of *P. aeruginosa*, except for the production of acid from mannitol, and nitrate reduction. Figure 1 shows the rod-like shape of strain YPJ80 on microscopic photographs.

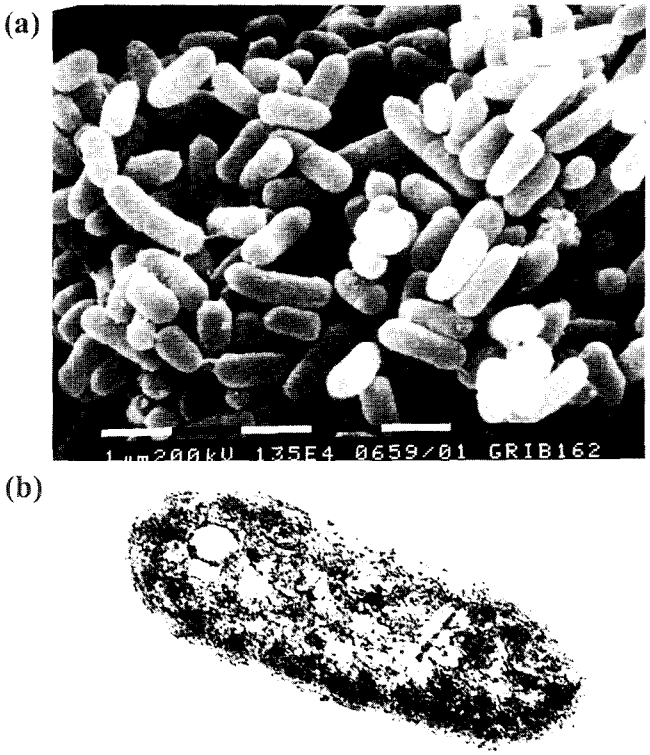

Fig. 1. Scanning electron microphotograph (a) and transmission electron microphotograph (b) of *P. aeruginosa* YPJ80.

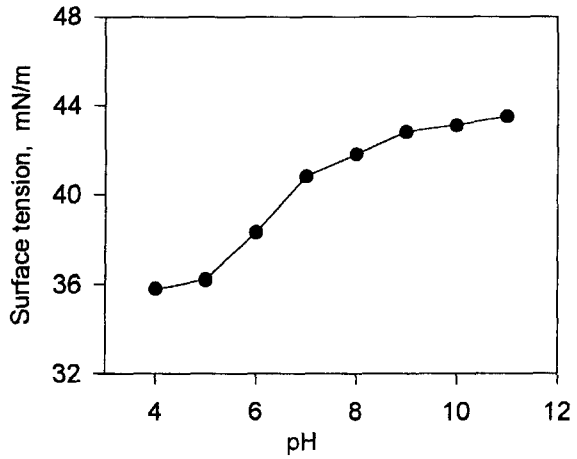

Fig. 2. Effect of pH on the surface tension of culture broth.

Cultivation was carried out on glucose medium at initial pH 7.0, 30°C, and 250 rpm for 2 days. Culture broth was diluted 4 times with distilled water.

Anionic Properties of YPJ 80 Biosurfactants

Ionic properties of the YPJ80 biosurfactants were investigated by measuring surface tension of culture broth in different pH conditions (Fig. 2). Culture broth was obtained after 2 days growth, and diluted 4 times with distilled water. Surface tension of broth was measured by adjusting the pH with 1 N NaOH and 1 N

HCl. As the solution became more acidic, surface tension became lower, which reveals that the surfactants from YPJ80 are anionic. It is similar to the results of previous reports that *P. aeruginosa* produces anionic surfactants [1, 9, 11].

Identification of Glycolipid

To investigate the type of YPJ80 biosurfactants, thin layer chromatography was carried out. Three major spots were detected on TLC. The component at R_f=0.93 showed positive detection under UV light and iodine vapor. The next two components (R_f=0.70 and 0.45) were detected using anthrone, iodine, rhodamine B reagent, indicating the presence of sugar, lipid, and free carboxylic acid [10, 11]. Thus, these two compounds were designated as glycolipid A and B (R_f=0.70 and 0.45), respectively.

Most biosurfactants of the glycolipid type are known to have hexose as a sugar component. One exception is rhamnolipid which has deoxyhexose as the hydrophilic structure in glycolipid. Figure 3 shows the spectrums of hexose (glucose) and YPJ80 biosurfactants after the reaction with thioglycolic acid. The shoulder around 430 nm indicates the presence of hexose. For YPJ80 glycolipids, no shoulder was observed around 440 nm. It means that YPJ80 glycolipids have deoxyhexose instead of hexose [2].

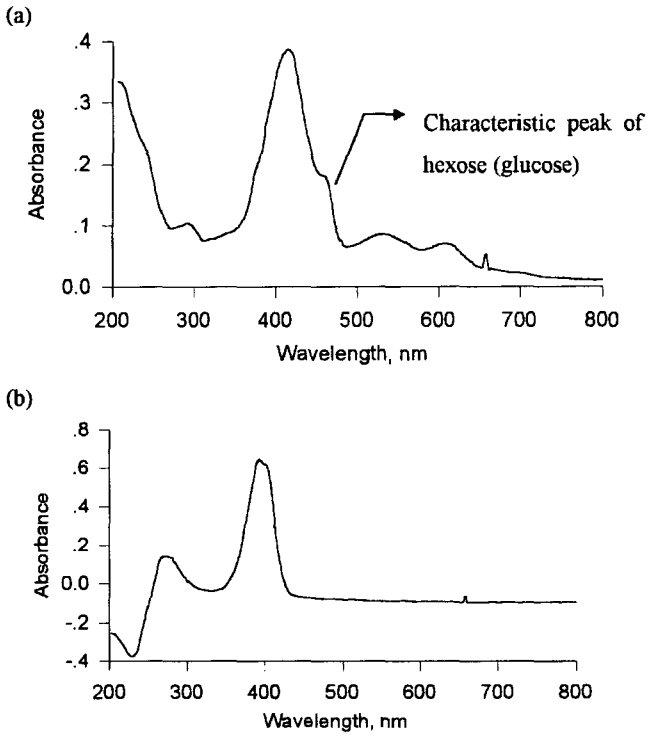

Fig. 3. Spectrum of glucose solution (a) and culture broth of glycerol medium (b) after reaction with sulfuric acid-thioglycolic acid.

Measurement of Surface Tension

Surface tension was measured to investigate critical micelle concentration (CMC) and minimum surface tension. Figure 4 shows the relationship between the concentration of biosurfactants and the corresponding surface tension. The minimum surface tension was 30.1 mN/m at pH 4.0. Upon the pH change of solution, CMC changed. At pH 4.0, CMC seems to be about 50 ppm, and about 500 ppm at pH 7.0 and 10. It means that more biosurfactants are needed to maintain the same surface tension at higher pH levels.

Emulsification of Oils

When oils are mixed with water, oils show a tendency to absorb water and form W/O emulsification. Such W/O emulsification results in the retardation of biodegradation by microorganisms. With surfactants such as biosurfactants, oils can be emulsified into water. This O/W emulsification accelerates biodegradation by enhancing bioavailability. The glycolipid biosurfactant produced from YPJ80 was examined in relation to the emulsification of hydrocarbons and oils including crude Arabian light oils.

Figure 5 shows the emulsification of oils and hydrocarbons by YPJ80 biosurfactants and emulsan. Emulsan was chosen as a control biosurfactant for the comparison of oil emulsification with YPJ80 biosurfactants for its representativity as bioemulsifier in the field of microbial enhanced oil recovery and oil clean-up. Emulsan could emulsify olive oil and soybean oil at 40 ppm, while no emulsification was observed with a hydrocarbon mixture and crude Arabian light oil. On the other hand, YPJ80 biosurfactants showed absolute emulsification

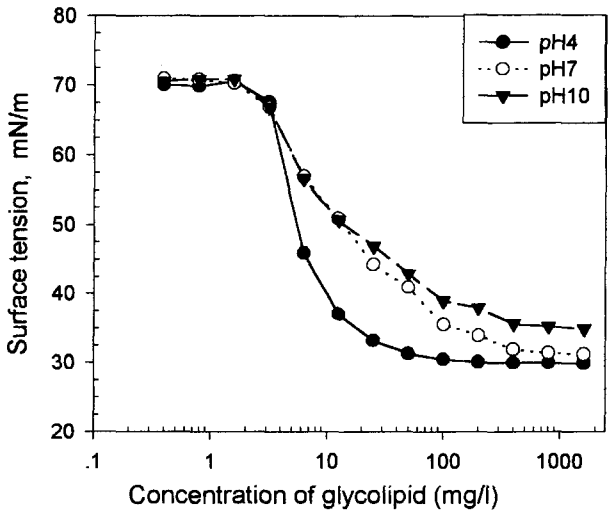

Fig. 4. Dependence of surface tension on the concentration of crude glycolipid.

Crude glycolipid was dissolved in 20 mM phosphate buffer (pH 7.0), and the pH was adjusted by 1 N NaOH and 1 N HCl.

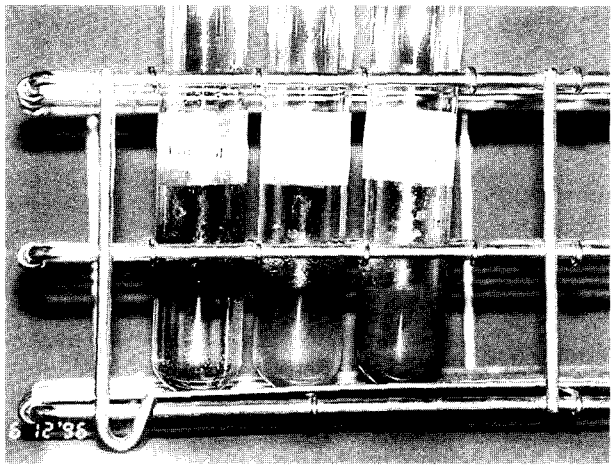

Fig. 5. Emulsification of crude Arabian light oil by partially purified glycolipid YPJ80 and emulsan at 400 mg/l, respectively. Crude oil was added at 1% (v/v).

Table 3. Comparison of emulsification against crude Arabian light oil and hexadecane.

Type of biosurfactant	Oils	Emulsifying activity (A_{620})	
		pH 6.0	pH 8.0
Emulsan (400 mg/l)	Hexadecane	0.65	0.73
	Crude Arabian light oil	0.73	0.89
Glycolipid YPJ80 (400 mg/l)	Hexadecane	3.57	1.79
	Crude Arabian light oil	3.20	3.33

regardless of types of hydrocarbons or oils. Quantitative comparison with emulsan is summarized in Table 3.

In conclusion, the glycolipid biosurfactants from *P. aeruginosa* YPJ80 could be effectively applied to the emulsification of oils, and is expected to be successfully used for the treatment of oil spills.

Acknowledgments

This work was performed through the financial support from Korea Science and Engineering Foundation. We thank Jeon, J.-S. for her excellent technical assistance.

REFERENCES

1. Arino, S., R. Marchal, and J. P. Vandecasteele. 1996. Identification and production of a rhamnolipid biosurfactant by a *Pseudomonas* species. *Appl. Microbiol. Biotehnol.* **45:** 162–168.
2. Chandrasekaran, E. V. and J. N. BeMiller. 1980. Constituent Analysis of Glycosaminoglycans, p. 94. *In* R. L. Whistler (ed.), *Methods in Carbohydrate Chemistry*, vol. **8**. pp. 95–96. Academic Press, NY, U.S.A.
3. Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams. 1986. *Bergey's Manual of Determinative Bacteriology*, Williams and Wilkins Co., Baltimore, U.S.A.
4. Kosaric, N., N. C. C. Gray, and W. L. Cairns. 1987. Introduction: Biotechnology and the surfactant industry, *In* N. Kosaric (ed.), *Biosurfactants and Biotechnology*, pp. 1–16. Marcel Dekker. NY, U.S.A.
5. Lang, S. and F. Wagner. 1987. Structure and properties of biosurfactants, *In* N. Kosaric (ed.), *Biosurfactants and Biotechnology*, pp. 21–41. Marcel Dekker. NY, U.S.A.
6. Lin, S. C. 1996. Biosurfactants: Recent advances. *J. Chem. Tech. Biotechnol.* **66:** 109–120.
7. Morikawa, M., H. Daiso, T. Takao, S. Murata, T. Shimonishi, and T. Imanaka. 1993. A new lipopeptide biosurfactant produced by *Arthrobacter* sp. Strain MIS38. *J. Bacteriol.* **175:** 6459–6466.
8. Muller-Hurtig, R., F. Wagner, R. Blaszczyk, and N. Kosaric. 1993. Biosurfactants for environmental control, *In* N.Kosaric (ed.), *Biosurfactants*, pp. 447–469. Marcel Dekker. NY, U.S.A.
9. Ramana, K. V. and N. G. Karanth. 1989. Factors affecting biosurfactant production using *Pseudomonas aeruginosa* CFTR-6 under submerged conditions. *J. Chem. Tech. Biotechnol.* **45:** 249–257.
10. Rendell, N. B., G. W. Taylor, M. Somerville, H. Todd, R. Wilson, and P. J. Cole. 1990. Characterization of *Pseudomonas* rhamnolipids. *Biochem. Biophys. Acta* **1045:** 189–193.
11. Robert. M, M. E. Mercade, M. P. Bosch, J. L. Parra, M. J. Espunny, M. A. Manresa, and J. Guinea. 1989. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. *Biotechnol. Lett.* **11:** 871–874.
12. Rosenberg, E., A. Zuckerberg, C. Rubinovitz, and D. L. Gutnick. 1979. Emulsifier of *Arthrobacter* RAG-1: Isolation and emulsifying properties. *Appl. Environ. Microbiol.* **37:** 402–408.